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5 **Cancer diagnosis by the measurement of Nup88 in body samples**

10 Field of the invention

This invention relates to the field of cancer diagnosis. Particularly, this invention deals with the diagnosis of the existence and/or the pathological developmental stage and/or the grade of malignancy of carcinomas and/or sarcomas in mammals.

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Background art

Mammalian cancer is the sixth most common cancer among women in the United States and constitutes the forth cause of death. Because of its asymptomatic early stage and the lack of an effective early-stage detection test, this cancer is the most lethal among all gynecological malignancies. Mammalian carcinogenesis involves multiple genetic changes, revealed as alterations in the expression of certain genes, particularly those related to cell cycle regulations, such as the tumor suppressor genes p53, p16, and BRCA, and the onco-
20 genes AKT2, ras, and c-Myc. Understanding the mechanisms of these changes could lead to using the genes as markers for the detection of mammalian tumors at an early stage.
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All previously known markers for cancer have certain drawbacks. Some stain only a small subset of malignancies, others do not allow to discriminate between the different patho-

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logical developmental stages of the corresponding disease, while others also stain not malignant tumors.

In 1993, a monoclonal antibody directed against candida krusei cytochrome c was shown to react with a cytoplasmatic fraction protein of human ovarian carcinoma cell line (Yasumoto K., *et al.*, 1993, Hum. Antib. Hybrid 4:186 ff.). Independently, it was reported that Nab C6, generated against candida albicans mannoproteins, reacted specifically with a 43kDa-molecule from a human mammalian carcinoma cell line, but not with non-neoplastic counterpart cells (Schneider J., *et al.*, 1996, Br. J. Cancer 77:1015-1020).

To sum up, it has been known in prior art that molecular antibody C6 crossreacts with some carcinoma cells but the target remained to be elusive. Accordingly, it is an object of the invention to provide for a new marker which is useful for cancer diagnosis.

Summary of the invention

In accordance with the purposes of this invention, as embodied and broadly described herein, this invention relates to a method of diagnosis of the existence and/or the pathological developmental stage and/or the grade of malignancy of carcinomas and/or sarcomas in mammals characterized by the preparation of a body sample of said mammals and the determination of the overexpression of the protein Nup88 in said sample.

This invention further provides a diagnostic kit for carrying out the method of the invention and a monoclonal antibody.

Detailed description of the invention

The invention is directed to a method of diagnosis of cancer, namely of all diseases belonging to the group of carcinomas and/or sarcomas of mammals. Carcinomas and/or sarcomas are epithelial tumors, epithelial tumors of the stomach, particularly infiltrating adenocarcinoma and *in situ* carcinoma, epithelial tumors of the colon, particularly infiltrating adenocarcinoma, *in situ* adenocarcinoma, villous adenoma, tubular adenoma, neuroendo-

- crine carcinoma, epithelial tumors of the liver, particularly hepatocellular carcinoma, dysplastic nodules, epithelial tumors of the pancreas, particularly adenocarcinoma, neuroendocrine carcinoma, epithelial tumors of the breast, particularly infiltrating ductal carcinoma, infiltrating lobular carcinoma, *in situ* ductal carcinoma, *in situ* lobular carcinoma, fibroadenoma, fibrocystic disease, epithelial tumors of the lung, particularly squamous carcinoma, adenocarcinoma, bronchioalveolar carcinoma, large cell carcinoma, neuroendocrine carcinoma, carcinoid, hyperplastic bronchi, epithelial tumors of the ovary, particularly cystadenoma, benign teratoma, borderline serous carcinoma, borderline mucinous carcinoma, serous carcinoma, mucinous carcinoma, endometrioid carcinoma, clear cell carcinoma, epithelial tumors of the uterus, particularly endometrial carcinoma, endometrial hyperplasia, epithelial tumors of the prostate, particularly adenocarcinoma, PIN high grade, PIN low grade, benign glandular hyperplasia, epithelial tumors of the kidney, particularly clear cell carcinoma, epithelial tumors of the adrenal, particularly cortical adenoma, mesenchymal tumors, particularly fibrosarcoma, malignant fibrous histiocytoma, Kaposi sarcoma, dermatofibrosarcoma, protruberous giant cell tumor (benign), leiomyoma, atypical fibroxanthoma, angioliipoma, miscellaneous tumors, particularly large cell lymphomas, lymphoblastic lymphomas, Hodgkin's disease, malignant mesothelioma, benign mesothelioma, glioblastoma multiforme, malignant melanoma.
- 20 The method of the invention is a diagnostic method for mammals, particularly those susceptible for veterinary or human medical care, preferably human beings. The method of the invention precludes the preparation of a body sample.
- The term *body sample* embraces but is not confined to blood, smears, sputum, urine, stool, *liquor cerebrospinalis*, bile, gastrointestinal secretion, lymphatic liquor and biopsies of organs to be tested,. All these body samples have to undergo a preparation procedure as known in the art for the determination of the overexpression of proteins. For instance, biopsies are cut into slices which are investigated by means of histology. Another example would be the examination of blood by well known ELISA techniques (*Enzyme Linked Immuno Sorbent Assay*).
- 30 The method of the invention relies in the determination of the overexpression of the protein Nup88 in said body sample. Nup88 was recognized by the inventors as the mammalian molecule to which the monoclonal antibody C6, generated against *Candida albicans* man-

noproteins crossreacted, when used to stain a tumor or mammalian carcinoma cell line (Nerea Martínez, Angel Alonso, María Dolores Moragues, José Potón, José Schneider, *Cancer Research* 59, 5408-5411, 1999, the disclosure of which is incorporated by reference). Nup88 ([GeneBank Y08612](#)) had been found to be associated with the central domain of CAN/Nup214, a nuclear pore complex component putatively implicated in the nuclear protein import, nuclear mRNA export, and the regulation of cell cycle (Van Deursen *et al.*, *Embo J.* 15:5574-5583, 1996). Notably, the CAN/Nup214 protooncogene is involved in chromosomal rearrangements related to two variants of leukemia (von Lindern M., *et al.*: 1992, *Mol. Cell. Biol.* 12:1687-1697; von Lindern M., *et al.*, 1992b, *Mol. Cell. Biol.* 12:3346-3355). The inventors showed by immunohistochemistry that a polyclonal antiserum directed to Nup88 recognized several human tumor cell lines as well as ovarian carcinomas in tissue sections; parallel results were obtained by immunoblot analysis (Martínez *et al.*, 1999). Taken together, the results disclosed in Martínez *et al.*, 1999 show that Nup88 is overexpressed in a series of tumor cell lines and in primary human ovarian tumors when compared with the corresponding tissue. Furthermore, now it has been found that overexpression of Nup88 in body samples is also indicative for the pathological developmental stage and/or the grade of malignancy of carcinomas and/or sarcomas. The term grade of malignancy is to be understood according to Roche Lexikon Medizin, 4th edition, Urban and Fischer, München, p. 909, col. 1, p. 1057, col. 1. The diagnosis of the pathological developmental stage is defined according to Roche Lexikon Medizin, 4th edition, p. 1582.

The diagnostic method of the invention is based on the determination of the overexpression of protein Nup88 in a body sample. The term overexpression means in the context of the invention that more protein Nup88 can be found in the body sample than in healthy controlled tissue. A person skilled in the art is aware, that assessing overexpression may also include the cytological and histological localization of Nup88 in the body tissue to discriminate against artifacts and to improve the signal to noise ratio of the determination procedure. The apparent cytological localization of Nup88 may also be dependent on the determination method employed. For instance, immunofluorescence analysis showed that Nup88 is located mainly in the nuclear membrane and to a minor extent in the cytoplasm.

In contrast, immunohistochemistry studies revealed a predominantly cytoplasmic accumulation of Nup88 in tumor cells (Martínez *et al.*, 1999, p. 5410, col. 2, Fig. 4).

The determination of the overexpression of the protein Nup88 is preferably performed using a *protein binding molecule* binding to Nup88, that means that the overexpression status is determined by measuring the amount of the protein, e.g. by use of an ELISA system or a biosensor. Such protein binding molecules may be natural antibodies such as polyclonal or monoclonal antibodies or recombinant antibodies such as chimeric proteins that exhibit homology to antibodies of mammals. The generation of said chimeric proteins is well-known in the art and described for instance in EP-B-0 368 684 and Little M., Kipriyanov S. M., Le Gall F, Moldenhauer G. 2000, Immunol. Today: 21(8):364-70. These chimeric proteins can be prepared by amplification of the variable or hypervariable regions of antibody genes of lymphocytes and the integration of these gene fragments in frame work vectors so as to produce all forms of recombinant antibodies. A particularly preferred protein binding molecule binding to Nup88 is the monoclonal antibody as deposited under the accession number given by the international depositary authority **DSM ACC2457** at DSMZ-Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany fulfilling the Budapest Treaty on the international recognition of the deposits of microorganisms for the purposes of patent procedure. This monoclonal antibody is particularly suited for the purposes of the invention because of its affinity and specificity. A further preferred protein binding molecule binding to Nup88 is a chimeric protein that exhibits homology to antibodies of mammals and which is further characterized in that at least one CDR region of said monoclonal antibody is virtually identical with the corresponding counterpart of 149/1/1 as deposited under DSM ACC 2457. The term CDR region is defined as known in the art, for instance in Little M., Kipriyanov S. M., Le Gall F, Moldenhauer G., Immunol. Today: 21(8):364-70, 2000 and in Suleyman S, Thompson KM, Mageed RA, Natvig JB, Scand J Immunol 2000 Oct;52(4):341-7 or in Takahashi M, Ueno A, Mihara H , Chemistry 2000 Sep 1;6(17):3196-203.

In a further embodiment of the invention, the overexpression of the protein Nup88 is determined by means of a *nucleic acid binding molecule* binding to the transcript of Nup88.

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It is well-known in the art, that overexpression of proteins is usually accompanied by the up-regulation of the production of the corresponding transcripts. Accordingly, the overexpression of the proteins to be investigated can be shown indirectly by measuring the amount of the corresponding transcripts. This can be done by the use of nucleic acid binding molecules binding to the transcript of Nup88. Such nucleic acid binding molecules are, for instance, oligonucleotides such as DNA-heteromers or RNA-heteromers comprising 7 to 40 monomeric units. The determination of overexpression of the protein Nup88 using nucleic acid binding molecules binding to the transcript of Nup88 can be performed using methods well-known in the art like polymerase chain reaction (PCR), including RT-PCR, hybridization techniques, including northern blot hybridization and other techniques suitable for the measurement of mRNA transcripts.

A further embodiment of the present invention is a diagnostic kit for carrying out the method of the invention comprising a protein binding molecule binding to Nup88. Preferably, the diagnostic kit also contains the protein Nup88 or an antigenic part thereof for control reactions.

Yet, another diagnostic kit for carrying out the inventive method comprises a nucleic acid binding molecule binding to the transcript of Nup88.

Another embodiment of the invention is a monoclonal antibody as deposited under DSM ACC 2457.

Brief description of the drawing

Fig. 1 (a) to (o) show immunohistochemical investigations of different tissues immunostained with polyclonal antiserum to Nup88.

Fig. 2 shows immunohistochemical investigations of other tissues using the same antiserum.

Fig. 3 shows an immunoblot analysis of Nup88 content in neoplastic, hyperplastic and normal tissues.

5 **Figure 1.** (a) to (o) show immunohistochemical investigations of different tissues immunostained with polyclonal antiserum to Nup88 using DAB as a chromomogen (DAB: 3,3',4,4'-tertraaminobiphenyl).

- 10 **1a)** Stomach adenocarcinoma; the prominent, somewhat hyperplastic mucosa (upper field) is not reactive whereas the carcinoma invading the submucosa (lower right) is clearly stained. X 60
- 1b)** Gastric adenocarcinoma; higher magnification of tumor in Fig. 1a. shows diffuse and intense immunostaining of carcinomatous glands. X480
- 1c)** Somach with extensive intestinal metaplasia; a strongly and diffusely stained in situ carcinoma is evident. X 180
- 15 **1d)** Colon adenocarcinoma; the normal mucosa on the left part of the field is negative while the basal aspect of the glands of a villous adenoma are moderately reactive (arrow). The overt carcinoma invading the submucosa is richly stained. X120
- 1e)** Colon adenocarcinoma; higher magnification of tumor in Fig. 1d shows diffuse, strong and granular reaction of malignant glands. X 620
- 20 **1f)** Colon, neuroendocrine carcinoma of colon. Note portions of characteristically organoid clusters diffusely reactive for Nup88. X 480
- 1g)** Liver, cirrotic septum in the liver that included the carcinoma depicted in 1h. Note reactivity in proliferating bile ductules (arrows) amidst non-reactive fibroconnective tissue. X180
- 25 **1h)** Liver, hepatocellular carcinoma; note strong, extensive and granular reaction. X620
- 1i)** Pacreas, adenocarcinoma of pancreas showing strong Nup88 reaction of malignant glands. X420

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1j) Breast, infiltrating ductal carcinoma of breast showing strongly Nup88 immunoreactive cell clusters. X 420

1k) Breast, fibrocystic changes of breast. Note negative hyperplastic and cystically dilated ducts. X120

5 **1l)** Breast, intraductal carcinoma of breast, cribriform variant. The reaction is diffuse but is best appreciated towards the periphery of the duct (arrows). X120

1m) Lung, well differentiated squamous carcinoma of lung; portion of neoplastic cluster showing strong staining particularly at the periphery of the aggregate; the central, more mature cells approaching pearl formation show but scanty reacting granules. X 620

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1n) Lung, moderately differentiated adenocarcinoma of lung; note strong and diffuse reaction. X480

1o) Bronchial carcinoid. Note moderately but diffusely reactive tumor ribbons and clusters amid the negative stroma. X 180

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Figure 2.- the same antibody was used as described for Fig. 1. The chromogene used was DAB except for Fig. 2n for which we used alkaline phosphatase (red).

2a) Ovary, moderately differentiated ovarian serous cystadenocarcinoma; note rich and diffuse reaction of the neoplastic papillae. X480

20 **2b)** Endometrium, well differentiated endometrial adenocarcinoma showing strong and diffuse Nup88 reactivity. X480

2c) Endometrium, cystic endometrial hyperplasia; no reaction is evident. X 180

2d) Prostate, prostatic adenocarcinoma, (Gleason 4+5); strong reaction involving the majority of the malignant cells is seen. X 420

25 **2e)** Prostate, periphery of prostatic carcinoma shown in Fig. 2d. Small clusters of malignant cells are stained; note also the overwhelming reaction of neoplastic cells around and within a nerve. X 540

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2f) Prostate showing variably hyperplastic and/or atrophic glands; no Nup 88 reaction is seen. X 180

2g) Kidney, renal cell carcinoma, clear cell type. A strong and extensive reaction is evident. X 480

5 **2h)** Soft tissue, malignant fibrous histiocytoma; a moderate but diffuse reaction involves most neoplastic cells. X480

2i) Leiomyosarcoma metastatic to lung (see Fig. 3, immunoblot B5). Strong reactivity is evident particularly in the bizarre giant cells. X620

10 **2j)** Lymph node, large cell lymphoblastic lymphoma diffusely reactive for Nup 88. X 160

2k) Pleura, malignant papillary mesothelioma richly and diffusely reactive for Nup88. X480

2l) Abdomen, benign cystic mesothelioma; no reaction is seen. X480

2m) Brain, glioblastoma multiforme; a rich and extensive reaction is evident. X480

15 **2n)** Skin, malignant melanoma using alkaline phosphatase as chromogen (red). A rich and diffuse reaction is noted. X480

2o) Fetal lung (20 weeks; 360g). A moderate but extensive reaction involves the primitive air spaces. X320

20 **Figure 3.** Analysis of Nup88 content in tumors and normal tissues by immunoblotting. Total protein extracts of colon (**A**), lung (**B**), breast (**C**) and ovary (**D**) were prepared as described in Methods and probed with the polyclonal antiserum to Nup88.

25 **A (colon):** lanes 1, 2, 3, 4 and 5 represent adenocarcinomas; lane 6 is the negative control consisting of normal human lymphocytes while lane 7 is the positive control representing an ovarian carcinoma known to expressed aduntan Nup88 from previous studies.

B (lung): lane 1 represents a bronchial carcinoid depicted in Fig, 1o. Lanes 2 and 4 represent an adeno- and a squamous carcinoma respectively while lane 3 is a poorly

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differentiated squamous carcinoma treated with chemo- and radio-therapy prior to removal. The strongly reactive lane 5 represents a metastatic leiomyosarcoma depicted in Fig. 2i, lane 6 represents hyperplastic bronchi in the vicinity of but not involved by a carcinoma. Negative control, lane 7, as in A.

5 **C (breast):** lane 1 represents typical, benign, fibrocystic changes depicted in Fig. 1k. Lanes 2 and 3 represent fibroadenomas; lanes 4 and 5 represent infiltrating ductal carcinomas, while lane 6 is an infiltrating lobular carcinoma. Lane 7 is a normal (control) adult female breast while lane 8 is a known, positive ovarian carcinoma.

10 **D (ovary):** lane 1 represents a benign mucinous cystadenoma, lanes 2 and 3 represent papillary serous carcinomas, and lane 4 represents a metastasis from a gastric signet ring cell carcinoma. Lane 5 is a surprisingly low signal given by an endometrioid carcinoma, Lanes 6, 7 and 8 represent negative controls while lane 9 is the positive counterpart.

15 For further details see the following Example which illustrates the invention without limitation.

Example

Material and Methods

Samples

Cases were selected on the basis of known diagnoses from the files of Rush-Presbyterian-Luke's Medical Center, Chicago, IL. Most of these cases had been
25 extensively studied and characterized in previous investigations. Autopsy samples from adult, and fetal tissues (stillborn) were obtained from RPSLMC. All surgical operations were performed with the appropriate, informed consents, and were based on well established therapeutic and/or diagnostic procedures. Autopsies were per-
30 formed based on legal permits. The anonymity of the patients was duly protected in all cases. A total of 266 samples, includingg 230 surgical tissues, 20 adult

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autopsy samples and 16 fetal autopsy selected samples were studied. One or 2 paraffin blocks per case were chosen; diagnoses were confirmed on conventional hematoxylin and eosin stained sections by 2 independent observers (VEG and AO). All tissues had been fixed in formalin and conventionally processed. Sections for immunostaining were cut at 4 μ m, set on coated slides and placed on a warmer at 60 C° for 1 hour; subsequently, they were deparaffinized in xylene and graded alcohols. No pretreatment with microwaves or enzymes was applied prior to exposure to the primary antiserum. Immunostaining was accomplished by the avidin-biotin-peroxidase method as originally outlined by Hsiu et al [Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. J Histochem Cytochem 29: 577-580, 1981]; commercial reagents were used (Dako Corporation, Carpinteria, CA). Best results were obtained when the antiserum was applied overnight in a humid chamber at 4 C° at a concentration of 1/500; the diluent was that provided in a commercial kit (Ventana Medical Systems, Tucson, AZ). Binding sites were visualized with 3,3'-diaminobenzidine (Aldrich Chemicals, Danvers, MA), in the case of melanocytic lesions, alkaline phosphatase (Dako) was used as chromogen. All sections were briefly counterstained with hematoxylin to improve nuclear visualization. As negative controls, slides were similarly processed but the primary antiserum was omitted. Staining intensity was rated as weak, moderate or strong; in heterogeneous cases, the rating of the predominant pattern was the one recorded. The extent of the reaction was defined by the percentage of reactive cells, and graded from negative (0) to 1+ to 5+ as previously described (Moll et al. , 1987, Am J Pathol 127:288-304).

Immunoblots

For immunoblot analysis, freshly obtained samples from surgical or autopsy material were placed in vials containing precooled isopentane, and snap frozen in liquid nitrogen. These samples were kept in a deep freezer at -80 C° until used. Tumors were homogenized in 2% SDS-0.14 M β -mercapthoethanol and then centrifuged over a Qias shredder (Qiagen, Germany) column to shear the DNA. The protein content of the samples was calculated with the DC protein assay system of Bio-Rad

and 80 µg of proteins were separated on 7 % polyacrylamide gels. Proteins were electrotransferred to PVDF membranes, blocked with nonfat milk in PBS and incubated with our polyclonal antiserum at a dilution of 1:2000. After washing, the membranes were reacted with a POD-labelled goat-anti rabbit, washed again, and the reacting bands revealed with the ECL system of Amersham (Amersham, UK).

Results

All malignant neoplasms stained convincingly; most reactions were moderate to strong, and 3+ to 5+ in extent; in the series studied, not a single malignant tumor could be said to be non-reactive. All in situ carcinomas were invariably positive as were, albeit less so, dysplastic lesions that did not reach the level of carcinoma in situ. Benign tumors showed variably extensive and generally weak immunoreactivity. Salient points are outlined in **Table I**.

Immunohistochemistry

EPITHELIAL TUMORS

Gastric carcinomas were extensively and strongly stained; the contrast was evident between the mostly negative overlying mucosa and the convincingly reactive invasive tumor (**Figs. 1a and 1b**). Reactions were similar in tumors showing variably differentiated glands as in cases showing a linitis plastica pattern of diffusely invasive single cells. One case included intestinal metaplasia with some dilated glands as well as carcinoma in-situ and infiltrating carcinoma; notably the metaplastic and dilated glands were negative while the in-situ carcinoma was strongly stained (**Fig. 1c**). The immunostaining pattern was characteristically punctiform; the granules were rather large as compared with the delicate dots seen in synaptophysin reactions (Wiedenmann *et al.*, 1986, Proc. Natl Acad Sci USA 83:3500-3504; Gould *et al.*, 1987, Am. J. Pathol 126:243-257). Focally, a perinuclear localization was evident but predominantly the distribution was diffuse through the cytoplasm (see below) ; the cell membrane was not reactive. This pattern was consistently repeated in all samples studied.

Colonic adenocarcinomas were strongly and diffusely reactive. Blocks of several cases included normal colonic mucosa, polyps with variable degrees of dysplasia and infiltrating carcinoma; in these cases, the normal mucosa was for the most part negative, the polyps showed variable degrees and extent of staining generally paralleling the degree of dysplasia while the infiltrating carcinomas were invariably strongly and extensively stained (**Figs. 1d** and **1e**). Two carcinomas of mixed exocrine/neuroendocrine phenotypes and 2 pure neuroendocrine carcinomas were similarly stained (**Fig. 1f**). In polyps not associated with carcinoma, villous adenomas stained stronger than their blander, tubular counterparts; foci of intramucosal carcinoma in the former reacted even more convincingly. Hepatocellular carcinomas were strongly stained. One of these developed in the background of a severe cirrhosis; in this case, the proliferating bile ducts noted in the cirrhotic septa showed sporadic, moderately reactive cells (**Fig. 1g**) while the adjacent carcinoma was diffusely and intensely reactive (**Fig. 1h**). Seven pancreatic adenocarcinomas of variable degrees of differentiation showed moderate to strong, and consistently diffuse reactions (**Fig. 1i**); two neuroendocrine carcinomas were similarly stained. Regardless of tumor phenotype, non-neoplastic exocrine and neuroendocrine cells in the vicinity of the tumors or entrapped within them were occasionally and focally reactive.

All infiltrating breast carcinomas of ductal (**Fig. 1j**) or lobular types reacted convincingly while non-neoplastic ducts nearby or trapped within them did not stain. Mucinous (colloid) carcinomas showed strong staining of the scanty malignant cells whereas the dominant mucous pools did not react. In several carcinomas, we noted the strongest staining in the peripheral, invasive edge of the tumors. Fibrocystic areas including cyst formation, ductal hyperplasia, adenosis, papillomas and apocrine metaplasia in the vicinity of carcinomas showed focal, weak to moderate staining while similar areas not associated with carcinoma ranged from minimally reactive to entirely negative (**Fig. 1k**). Regardless of the presence of a synchronous carcinoma, significantly atypical ducts and ductal carcinomas in situ of all types reacted convincingly (**Fig. 1l**).

Pulmonary squamous, adeno-, bronchioloalveolar, large cell and neuroendocrine carcinomas of all types stained richly and extensively (**Fig. 1m** and **1n**); notably,

hyperplastic but non-neoplastic bronchi in the vicinity of and within tumors were often strongly stained. All bronchial carcinoids studied stained diffusely; the intensity of the reactions varied; moderate staining predominated but focally strong staining was occasionally noted (**Fig. 1o**).

5 Ovarian carcinomas including serous, mucinous, clear cell and endometrioid types were convincingly stained; borderline tumors stained moderately while their invasive (**Fig. 2a**), and particularly their high grade counterparts were intensely reactive. Benign cystadenomas and cystadenofibromas showed uneven, weak to moderate reactions. In a benign teratoma, cartilagenous cells, the basal cells of skin ad-
10 nexa and respiratoty-type epithelium showed moderate reactions. Endometrial carcinomas including endometrioid, clear cell and serous variants reacted strongly and extensively. Several of these cases were associated with endometrial hyperplasia; interestingly, while the carcinomas stained strongly, the bland variants of hyperplasia did not (**Figs. 2b** and **2c**). Also interesting was that the superficial, luminal aspect of several tumors stained less intensely than the deeper, invasive portion.

15 All prostatic carcinomas reacted strongly and diffusely irrespective of degree of differentiation (**Fig. 2d**) while non-neoplastic ducts and acini entrapped within them stained weakly and sporadically or not at all. In the case of poorly differentiated and hypernephroid carcinomas, single reactive cells were readily detected as were
20 minute clusters around or within nerves (**Fig. 2e**). Several carcinomas included convincing foci of PIN (prostatic intraepithelial neoplasia) which were convincingly stained. Benign, hyperplastic and atrophic glands were either negative (**Fig. 2f**) or showed rare, positive cells, most often in the vicinity of carcinoma. All renal carcinomas were of clear cell type and all reacted strongly (**Fig. 2g**); some reactive
25 renal tubules in the vicinity stained as well. Adrenocortical adenomas showed weak but fairly extensive reactions.

MESENCHYMAL TUMORS

30 Fibrosarcomas, malignant fibrous histiocytomas (**Fig. 2h**), and Kaposi sarcomas reacted strongly and extensively; a single leiomyosarcoma reacted strongly in both

primary and metastatic sites (**Fig. 2i**). Dermatofibrosarcoma protruberans, infantile fibrosarcomas, benign giant cell tumors, atypical fibroxanthomas and angioliomas showed decreasingly extensive and moderate to weak reactions. Two (uterine) leiomyomas stained rather extensively but weakly.

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MISCELLANEOUS TUMORS AND FETAL TISSUES

Diffuse large cell lymphomas stained strongly and diffusely as did a lymphoblastic lymphoma (**Fig. 2j**). In several cases of Hodgkin's disease, the Reed-Sternberg and the lacunar cells stained convincingly whereas the associated non-neoplastic leukocytes did not. Malignant mesotheliomas stained strongly (**Fig. 2k**); similar reactions were noted in epithelioid, sarcomatoid and biphasic variants. In contrast, samples from benign cystic mesotheliomas (multiple peritoneal inclusion cysts) were consistently negative (**Fig. 2l**). High-grade glioblastomas stained strongly and extensively (**Fig. 2m**). In-situ malignant melanomas showed convincing staining of the dysplastic melanocytes at the base of the epidermis, and of single cells migrating upwards in the epidermis. In invasive melanomas of various sites, reactions were diffuse and strong, and the coexistence of melanin and immunoreactive granules was clearly detectable in numerous cells (**Fig. 2n**); reactions were indistinguishable in epithelioid aggregates and in sarcomatoid foci.

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Fetal samples were included in the study based primarily on the state of tissue preservation. Reactions were particularly convincing in developing bronchi and primitive air spaces (**Fig. 2o**) as well as in the crypts of the colonic mucosa.

Immunoblots

The results of these experiments are shown in **Fig. 3**. In all samples of malignant tumors analysed, a clear increase in the amount of Nup88 was observed. All colon carcinomas showed reactive bands that were far stronger than the corresponding controls (A-6); the comparatively weak band noted in A-2 may reflect the tumor's extensive necrosis. In the case of lung tumors, the respectable band seen in the bronchial carcinoid (B-1) corresponds in fact to a rather intensely reactive tumor

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(Fig. 1o). Notice also the strong B-2 and B-4 bands (carcinomas) and compare with the relatively meager B-3 lane that represents a carcinoma treated with radiation and chemotherapy resulting in extensive tumor necrosis prior to surgical removal: The strong B-5 band corresponds to a leiomyosarcoma metastatic to the lung (Fig. 2i), and the weak but distinct B-6 corresponds to bronchi showing epithelial hyperplasia and metaplastic changes in the vicinity of a carcinoma.

The contrast between benign and malignant proliferations in the same organ is well exemplified in the series of breast lesions wherein all carcinomas (C-4, C-5 and C-6) show prominent bands while fibroadenomas (C-2 and C-3) and one bland variant of fibrocystic disease (C-1, corresponds to Fig. 1k) show delicate bands, and the sample of normal breast is virtually negative (C-7). In the case of the ovary, the meager band noted in D-1 (benign cystadenoma) offers a stark contrast with the broad bands seen in the carcinomas (D2, D3 and D4). Other than problems related to sampling, we cannot explain the weak band of the endometrioid carcinoma (D5) that by immunohistochemistry was moderately but convincingly decorated.

Densitometric quantification of the blots showed an increased expression in carcinomas between 1,5 and 5 times as compared with normal controls. However, it should be considered that these figures most probably represent a significant underestimation given that they do not take into account the considerable amounts of stroma that many of these tumors had. More detailed quantitative analyses should be performed based on larger pools of samples, and subsequent to appropriate microdissection.

Discussion

Our immunohistochemical results strongly reinforced by data from immunoblots of selected samples point to Nup88 as a molecule of exceedingly wide distribution that is consistently overexpressed in a broad spectrum of carcinomas, as well as in malignant mesotheliomas, many sarcomas, melanomas, gliomas and some lymphoreticular tumors. Nup88 expression was similarly enhanced in severe dysplasias and in situ carcinomas of organs such as colon, stomach, breast and prostate. We also

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showed distinct Nup88 enhancement during fetal development in sites including primitive pulmonary air spaces and colonic crypts. Focal expression was also noted in proliferative-reparative tissues in the vicinity of tumors, e.g., hyperplastic bronchial mucosa. Conversely, Nup88 was either sporadic or not detectable in most benign tumors and hyperplasias. In normal adult tissues, Nup88 was noted sporadically in sites such as colonic crypts, bronchial mucosa and fallopian tubes.

With regard to epithelial cancers, Nup88 enhancement was seen not only across a broad spectrum of sites but through all major differentiation lines. Thus, lung carcinomas with squamous, glandular and neuroendocrine features were strongly positive. In the GI tract and pancreas adeno- and neuroendocrine carcinomas were similarly positive, while in the ovary, serous, mucinous, endometrioid and clear cell carcinomas reacted as well. In addition, as noted earlier (Martínez et al, 1999), we also found that in sites such as the ovary, high grade carcinomas seemed to react more vigorously than their low grade or borderline counterparts. Furthermore, in some breast and endometrial carcinomas, we noted that the invasive periphery of the tumors stained more strongly than the center. The small number of cases studied to-date do not permit definite conclusions; yet, the potential significance of these observations merits re-exploration not only in the ovary but in other tumor systems. Also, interesting is that Nup88 overexpression seems to be independent of site of origin or presumed histogenesis as it was found in carcinomas from diverse organs derived from all embryonal layers.

Nup88 enhancement was clearly evident in severe epithelial dysplasias and in situ carcinomas of the colon, stomach, breast and prostate. This overexpression was evident irrespective of the presence or absence of an overt, synchronous cancer. Conversely, benign variants of conditions such as fibrocystic disease of the breast, endometrial hyperplasia, tubular adenomas of the colon and prostatic hyperplasia showed for the most part no significant Nup88 enhancement. Similar contrasts were noted in mesenchymal and other miscellaneous malignancies, i.e., strong reactions in a leiomyosarcoma and in malignant mesotheliomas contrasting with weak or absent staining in their benign counterparts. Notably, certain active reparative lesions, e.g., bile duct proliferation in cirrhosis associated with carcinoma,

and proliferating renal tubules in the vicinity of renal carcinomas associated with pyelonephritis showed focal but convincing Nup88 overexpression. Interestingly, such areas are known to undergo active remodelling as reflected by the enhancement of pertinent matrix molecules, e.g. tenascin, cellular fibronectins (Howeedy et al., 1990, Lab Invest 63:798-806; Gould et al, 1992, Lab Invest 67:71-79). These findings indicate that enhanced Nup88 reflects a certain selective cellular proliferation that is most often but not exclusively associated with the malignant or pre-malignant phenotypes.

Some of the above findings of Nup88 overexpression in malignancy coupled with its detection in some fetal tissues suggest some parallels between it and a number of onco-developmental marker molecules including CEA and related substances. However, the latter molecules are for the most part membrane-associated glycoproteins known to or suspected of subserving cell-cell adhesive functions (for overview and ref's see Koukoulis et al., 1998, Hum Pathol 30:1273-1275; Gould and Gould, 1999, Hum Pathol 30:1273-1275). In addition, they are selectively expressed in some epithelia but are virtually absent in others, and in non-epithelial tissues. Moreover, the characteristically punctiform perinuclear and cytoplasmic localization of Nup88 differs substantially from that of the above molecules. These observations added to those that Nup88 is also significantly enhanced in malignancies as diverse as carcinomas, some sarcomas and lymphomas, mesotheliomas, melanomas and gliomas point to significant differences between Nup88 and oncodevelopmental markers currently used.

A consistent finding in the present study was the predominantly cytoplasmic location of the overexpressed protein in the involved cells. Our previous studies on several cell lines showed that most of the protein was located at the nuclear membrane with comparatively small amounts in the cytoplasm. Noteworthy, in many neoplastic and some non-neoplastic cells is the presence of aggregates of annulate lamellae (Ghadially FN: Ultrastructural pathology of the cell and matrix. 1997, 4th ed, Butterworth-Heinmann, Boston, USA); and, these structures are thought derive from nuclear membranes (Kessel, 1983, Internatl Rev Cytol 82:181-305), and show features of the latter e.g. components of nuclear pore complexes including nucleo-

porins as described in *Xenopus* oocytes (Cordes et al, 1995, Eur J Cell Biol 68:250-255), and rat cells wherein they were visualized as cytoplasmic dots (Ewald et al., 1996, J Cell Sci, 109, 1813-24). Therefore, we speculate that the conspicuous cytoplasmic granules we found might reflect increased numbers of annulate lamellae.

5 In this context, it merits mentioning that other oncoproteins may also be aberrantly located, e.g., the known nuclear-cytoplasmic mislocation of the BRCA 1 gene product in breast carcinomas (Chen et al., J Biol Chem 271:32863-8).

The polyclonal antiserum applied in these experiments allowed us to recognize

10 clearly and consistently a Mr 88,000 band in immunoblot experiments. In addition, other weakly, reactive bands were at times detectable. The precise nature of these additional reactive bands remains unclear, but it should be stated that our antibody reacts strongly with the GST-Nup88 fusion proteins used for the immunization of the rabbits (Martínez et al., 1999). Also significant is that we cannot as yet state

15 whether the aforementioned bands of lower molecular weight reflect degradation products of a single molecule or additional, separate proteins that share a similar or a common epitope. The eventual identification of the epitope recognized by our antibody should help elucidate the fragment of the protein recognized by it. In our current data, some questions may be said to persist as to whether the material recognized by our antiserum corresponds in fact to Nup88. Significantly, in carcinomas of various sites, and in one sarcoma, strong and extensive immunostaining of tissue sections was paralleled by similarly strong M_w 88,000 reactive bands in western blots of the same samples; conversely, in samples of hyperplasias, benign tumors, and normal tissues, scanty, weak or absent tissue staining was reflected by

20 weakly reactive bands in the corresponding immunoblots. Moreover, no differences in intensity were noted in the additional, reacting bands found in tumors as well as controls thus reinforcing the notion that our antiserum is specific for Nup88. These data strongly suggest that the molecule in question corresponds indeed to Nup88.

In vertebrate cells, the nuclear pore complex (NPC) (for recent overview and ref's

30 see Allen et al., 2000, J Cell Sci 113:1651-1659; Blobel and Wozniak, 2000, Nature 403:835-836) is a large macromolecular aggregate with an estimated molecular mass of 125 MDa (Reichelt et al., 1990, J Cell Biol 110:883-894); it includes 50-

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100 proteins termed nucleoporins (Fountura et al., 1999, J Cell Biol 144: 1097-1112). Conversely, in yeast, NPC are smaller, have a molecular mass in the range of 66 MDa, and may include 30-40 nucleoporins (Rout and Blobel, 1993, J Cell Biol 123, 771-783). Among the known nucleoporins, the rather recently characterized Nup 88 in its dynamic subcomplex association with the oncogenic nucleoporin CAN/Nup214 seems to play essential roles; depletion of the complex results in defective import-export processes, and eventual cell cycle arrest (Fornerod et al., 1997, EMBO J 16:807-816); and, in overexpressing cells, it appears that CAN/Nup214 and one of its interacting proteins, i.e. Nup88, may function on both faces of the NPC (Boer et al., Exp. Cell Res 232, 182-185). Notably, preliminary experiments in our laboratory showed no increased expression of CAN/Nup214 in our tumor samples thus suggesting an uncoupling of the former from Nup88 at least in some instances. Interesting in this context is the fact, that the Nup98 gene seems to be involved in therapy-related leukemias by a translocation producing fusion proteins that may act as transcription factors modulating expression of other genes (Nishiyama et al., 1999, Genes Chromosomes Cancer 3, 215-220).

We can only speculate about the possible role (s) of Nup88 in malignant cells. A possible explanation is that its overexpression is simply the result of increased nucleocytoplasmic transport required to meet the growing demand of proteins by transformed cells. Increased traffic is indeed known to occur; in this context, it merits mention that the diameter of the pore channel in transformed mammalian cells is known to be increased (Feldherr and Aikin, 1995 Membr Prot Trans 2:237-259). An alternative explanation is that Nup88 may play a role in the formation and maintenance of anullate lamellae as outlined above; but while this might explain their presence it would not clarify their function.

Our findings suggest that this molecule may be a potentially significant marker given its dramatic overexpression in a broad spectrum of malignant tumors of literally all denominations. If these results were confirmed, Nup88 might be said to approach an ideal, generic marker of transformation readily demonstrable on conventional tissue sections, and possibly in cytologic samples.

TABLE I.

Nup88 Immunoreactivity of Tumors & Related Conditions

Site and Diagnosis	# of Cases & Reaction	¹ Extent of Reaction	² Intensity of Reaction
EPITHELIAL TUMORS			
STOMACH			
Infiltrating adenocarcinoma	11/11	3+/5+	m/s
In situ carcinoma	2/2	3+/4+	m/s
COLON			
Infiltrating adenocarcinoma	12/12	3+/5+	m/s
In situ adenocarcinoma	3/3	2+/4+	m
Villous adenoma	3/3	2+/3+	m
Tubular adenoma	5/5	2+/3+	w/m
Neuroendocrine carcinoma	2/2	3+/4+	m/s
LIVER			
Hepatocellular carcinoma	4/2	3+/5+	m/s
Dysplastic nodules	2/2	2+/3+	w
PANCREAS			
Adenocarcinoma	7/7	3+/4+	m/s
Neuroendocrine carcinoma	3/3	3+/4+	m/s
BREAST			
Infiltration ductal carcinoma	14/14	3+/5	m/s
Infiltrating lobular carcinoma	12/12	3+/5+	m/s
In situ ductal carcinoma	16/16	3+/4+	m
In situ lobular carcinoma	5/5	3+	m
Fibroadenomas	2/5	1+	w
Fibrocystic disease	16/28	1+/3+	w/m
LUNG			
Squamous carcinoma	8/8	3+/5+	m/s
Adenocarcinoma	12/12	3+/5+	m/s
Bronchoalveolar carcinoma	2/2	3+/4+	m
Large cell carcinoma	3/3	3+/5+	m/s
Neuroendocrine carcinoma	14/14	3+/5+	m/s
Carcinoid	9/9	2+/4+	m
Hyperplastic bronchi	3/3	2+/3+	w/m
OVARY			

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Cystadenoma	2/3	2+	w/m
Benign teratoma	1/1	2+	w/m
Borderline serous carcinoma	2/2	2+/3+	m
Borderline mucinous carcinoma	2/2	2+/3+	m
Serous carcinoma	6/6	3+/5+	m/s
Mucinous carcinoma	4/4	3+/5+	m/s
Endometrioid carcinoma	1/1	4+	m/s
Clear cell carcinoma	2/2	3+/5	m/s
UTERUS			
Endometrial carcinoma	12/12	3+/5+	m/s
Endometrial hyperplasia	4/10	0/2+	w
PROSTATE			
Adenocarcinoma	11/11	3+/5+	m/s
PIN, high grade	6/6	2+3+	m/s
PIN, low grade	2/4	1+/2+	w/m
Benign glandular hyperplasia	2/6	(+)/2+	w/m
KIDNEY			
Clear cell carcinoma	4/4	3+/5+	m/s
ADRENAL			
Cortical adenoma	2/2	2+/3+	w
MESENCHYMAL TUMORS			
Fibrosarcoma	5/5	3+/4+	m
Malignant fibrous histiocytoma	7/7	3+/4+	w/m
Kaposi sarcoma	2/2	3+/4+	m
Dermatofibrosarcoma	5/5	3+/4+	m
Protruberous			
Giant cell tumor, benign	0/2	0	-
Leiomyoma	2/2	3+/4+	w
Atypical fibroxanthoma	2/2	2+/3+	w
Angiolipoma	1/2	(+)	w
MISCELLANEOUS TUMORS			
Large cell lymphoma	3/3	3+/4+	m
Lymphoblastic lymphoma	1/1	3+/4+	m
Hodgkin's disease	4/4	2+/3+	m
Malignant mesothelioma	5/5	3+/5+	m/s
Benign mesothelioma	0/2	0	-
Glioblastoma multiforme	4/4	3+/5+	
Malignant melanoma			m/s
Infiltrating	4/4	3+/5+	m/s
In situ	2/2	3+/4+	m/s

(+):	< 1 % positive cells
1+:	1-5 % positive cells
2+:	6-15 % positive cells
2+:	16-50 % positive cells
4+:	51-95 % positive cells
5+:	95 % positive cells
w:	weak
m:	medium
s:	strong

Ins
C1

[illegible]